Detection of New Spider Toxins From a *Nephilengys borbonica* Venom Gland Using On-Line μ-Column HPLC Continuous Flow (FRIT) FAB LC/MS and MS/MS

Yasuhiro Itagaki,[1*] Tsuyoshi Fujita, Hideo Naoki,[1] Tadashi Yasuhara,[2] Marta Andriantsiferana,[3] and Terumi Nakajima[1]

[1]Suntory Institute for Bioorganic Research, Osaka, Japan
[2]Department of Nutrition Junior College of Tokyo University of Aguriculture, Tokyo, Japan
[3]Faculty of Science University of Antanarivo, Antanarivo, Madagascar

ABSTRACT Spider venom glands store various novel neurotoxic acylpolyamines which present potent and irreversible inhibition of the glutamatergic synapses. We have developed a new highly sensitive analytical method to detect and characterize structures of those neurotoxins stored in a single venom gland using micro bore column high performance liquid chromatography (HPLC)-continuous flow FRIT fast atom bombardment mass spectrometry (HPLC-FAB/MS) and tandem mass spectrometry (MS/MS) array detection system. The high-energy collision induced dissociation (CID) spectra of sodium cationized spider toxins produced very strong structurally informative product ions which afforded the location of nitrogen atoms and the connectivity of methylene units within the polyamines. This methodology permitted detection of 40 amino acid containing acylpolyamines in the venom of *Nephilengys borbonica* from which structures of only five compounds were previously known. *Nat. Toxins 5:1–13, 1997.*

Key Words: spider venom; acylpolyamines; liquid-chromatography/mass spectrometry; mass spectrometry/mass spectrometry (MS/MS); charge remote fragmentation

INTRODUCTION

Spiders and other venomous arthropods store various complex neuroactive chemical weapons in their own venom glands which are able to paralyze and kill other arthropods. These venom toxic compounds often exhibit specific biological activities, and some of them have been used as tools in the neurochemical research. With the progress of isolation and bioassay methods in very reduced scale, spiders are receiving very close attention in the search for unique neurotoxic substances. Besides proteins and polypeptides, a series of low molecular weight glutaminergic blocker acylpolyamines, like JSTX (1–4), nephilatoxins (1–12), argiopinins (I–V), etc., were found in spider venom glands [Aramaki et al., 1987a,b; Toki et al., 1988, 1990; Skinner et al., 1990; Jasys et al., 1990; McCormick et al., 1993; Quistad et al., 1990, 1991].

For isolations and structure determinations, high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), chemical ionization (CI), and FAB/MS are used extensively. However, these procedures require a number of venom glands and even then minor neurotoxic substances may not be detected.

Since, throughout the world, more than 30,000 species of spiders from over 70 different families had been described by the early 1980s [McCormick and Meinwald, 1993], it is necessary to find a more efficient way to study the structures and biological activities of those neurotoxic substances, if possible by the use of a single venom gland.

It has been shown that direct coupling a HPLC to MS and LC/MS/MS techniques, especially continuous flow FRIT FAB LC/MS , developed by Ito et al. [1985] and Caprioli et al. [1986] have excellent potential for handling such trace level complex mixtures. Packed μ-column HPLC could be coupled with a mass spectrometer using FRIT-FAB interface without postcolumn splitting because of its low flow rate [Kassel et al., 1991a,b]. Pleasance and co-workers reported the effect of matrix additions to the mobile phase on the chromatographic separation and designed the post column FAB matrix addition system [Pleasance et al., 1990] that could maintain the polarity and viscosity of the mobile phase and permit the use of a UV detector before the introduction of samples into the mass spectrometer, thus avoiding the UV signals of the employed matrix. FAB mass spectra of spider toxins showed a characteristic $(M+H)^+$ ion, but lacked

*Correspondence to: Yasuhiro Itagaki, Suntory Institute for Bioorganic Research, Wakayamadai, Shimamoto-cho Mishima-gun Osaka, 617 Japan.

Received 1 August 1996; accepted 10 October 1996.

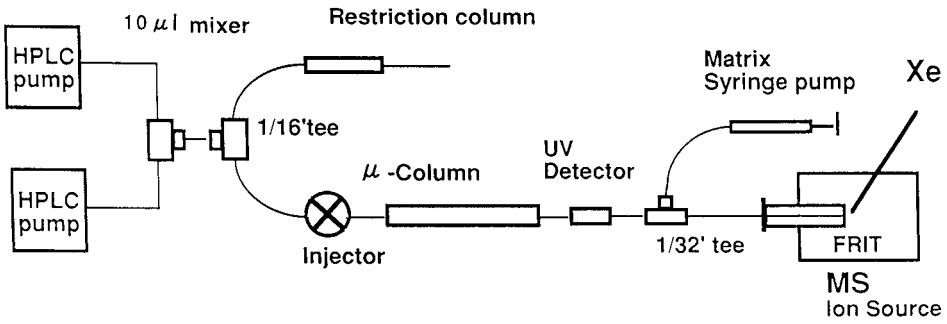

Fig.1. Schematic diagram of μ-column HPLC/FRIT-FAB system. The microflow cell for the UV detector was prepared from 0.32 mm i.d. fused-silica by removing polyamide coating. Stainless steel capillary tubing of 0.05 mm i.d. × 0.3 mm o.d. was glued into the fused-silica tubing with epoxyresin adhesive.

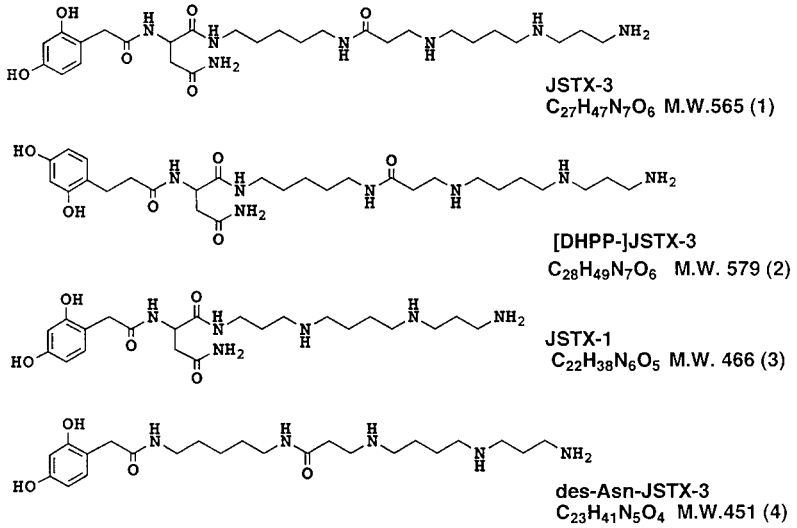

Fig. 2. Model compounds used for system sensitivity check.

sufficiently intense fragment ions required for structure analysis. The most commonly used technique is the CID of a precursor ion with a neutral He gas, but effective structure information can be obtained with the attenuation of the precursor ion intensity to ca. 30% such that only some portion of the precursor ions are used [Kim and McLafferty, 1978]. The use of an array detector with four sector tandem MS/MS allows detection of even lower yield product ions at few picomolar level of samples [Cottrell and Evans, 1987]. We have reported [Fujita et al., 1995] on the fragmentations of $(M+H)^+$ and sodium adduct $(M+Na)^+$ ions of acylpolyamines using four sector tandem MS/MS and proved that sodium adduct ions formed much more intense structure related information with the effect of charge remote fragmentation [Adams and Gross, 1987; Adams 1990; Ann and Adams 1993].

In this paper, we report the analytical results of spider toxins extracted from a single *Nephilengys borbonica* venom gland with the use of μ-column HPLC-continuous flow FRIT-FAB LC/MS and MS/MS array detection system combined with the post column matrix addition method.

MATERIALS AND METHODS

Collection of Spider Venom Gland and Extraction

Spiders *(Nephilengys borobonica)* were collected from Madagascar. Venom glands were taken out and kept in a deep freezer (at −80°C). Each venom gland (1.39 mg) was homogenized in 2 ml of 60% acetonitrile/H_2O containing 0.1% trifluoroacetic acid (TFA) using glass Teflon homogenizer (Iuchi, Osaka, Japan) and centrifuged at 14,000 rpm/min for one hour. The supernatant fluid was filtered through Microcon 3 (Amicon, Inc. Beverly, MA) to eliminate materials over 3,000 daltons (Da). The filtrate was diluted to 1/10th the original concentration with water and was used for the LC/MS and MS/MS measurements without any further treatment.

LC Systems

The basic experimental set up used is shown in Figure 1. Two LC 110 P model pumps (Yokogawa, Tokyo, Japan) delivered solvents to a gradient solvent mixer (10 μl). After

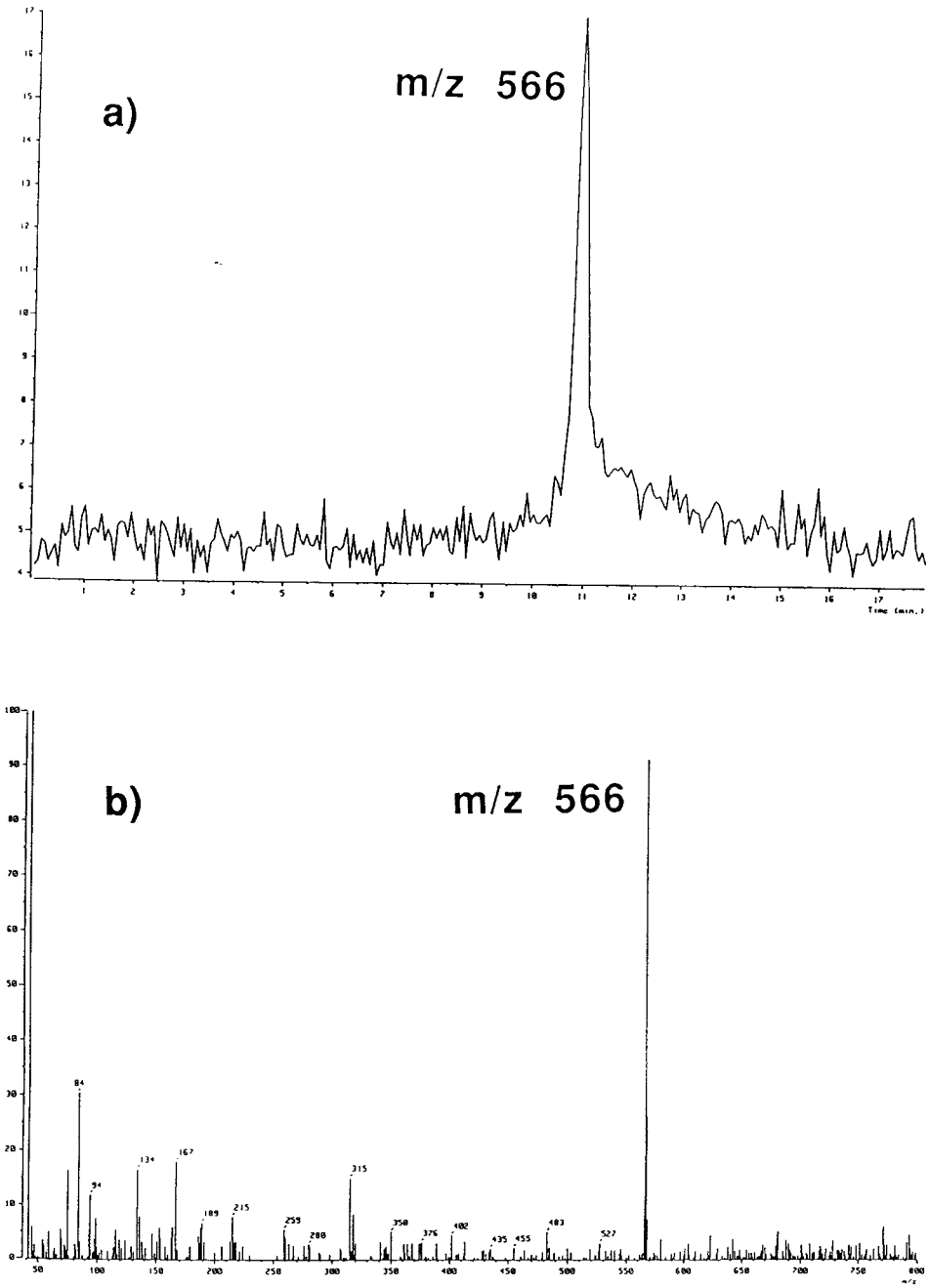

Fig. 3. a: Mass chromatogram of JSTX-3 protonated molecular ion. **b:** FRIT FAB-MS spectrum of JSTX-3 (2 pmol/μl: 10 μl injection).

mixing the solvents, the gradient flow was split into 200 to 1 using a restriction column (ODS-5. 45 × 150 mm, Nomura Chemical Co., LTD, Seto-shi, Japan) prior to introduction to the valve injector. The samples, injected through a conventional Reodyne sample injector (Model 7125) equipped with a 10 μl injection loop, flowed onto a 0.3 × 150 mm Develosil ODS-HG-5 μ-column (Nomura Chemical Co., LTD). Samples were concentrated at the head of the μ-HPLC column by the isocratic use of water [Nagae and Takeuchi, 1991]. Components eluted under gradient conditions, flew through a 60 μm (i.d.) fused silica capillary and through a Yokogawa 100U UV detector outfitted with a through-the capillary optical cell operated at 214 nm. After UV detection, flowing through a 60 μm (i.d.) fused silica capillary again and mixing at the Valco ZT.5(1/32)T with the matrix solution, the mixture was pumped out by a syringe pump (Harvard Apparatus Model 22 Southnatick, MA) and was injected into a JEOL FRIT-FAB interface at the flow rate of 4 μl/min. Solvent A) H_2O/TFA = 100/0.1 [v/v] and B) (CH_3CN/TFA = 100/0.1 [v/v] were prepared and used for

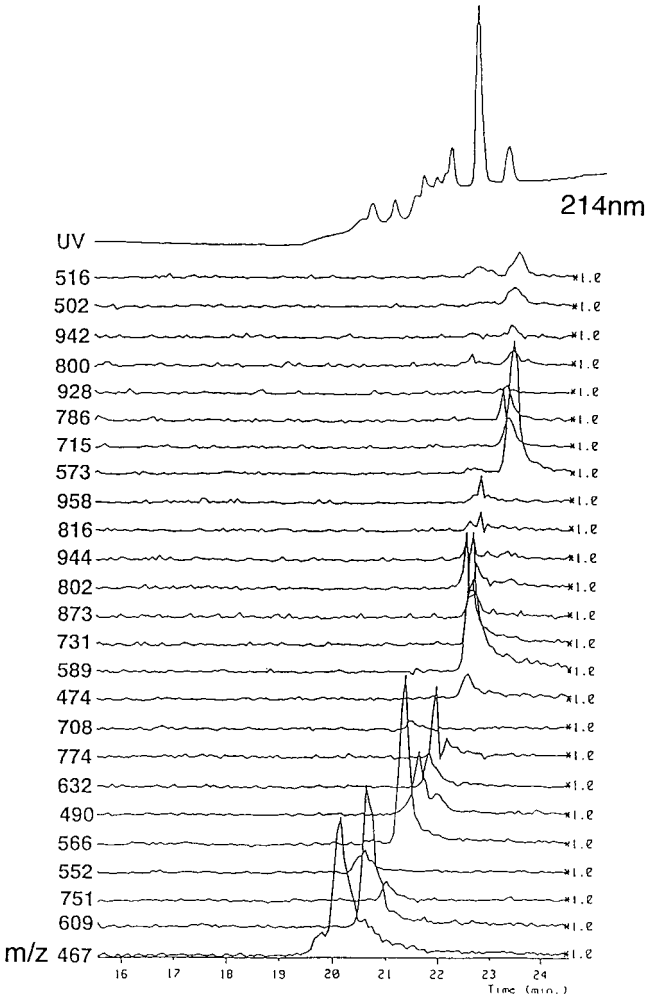

Fig. 4. UV and FAB mass chromatograms of major constituents stored in the venom gland.

HPLC. As a standard procedure, solvent A 100% was kept isocratic for 5 min then after a linear gradient of solvent B (0–80%) for 20 min was applied. Glycerol matrix (0.5% glycerol, 0.5% TFA, 60% MeOH/H_2O solution) was added at the flow rate of 0.8 μl/min using the postcolumn addition system. UV spectra were recorded using a Hewlett Packard HPLC model 1090 with an array detector. The solutions of venom extracts (20 μl) were loaded onto a Cosmosil $5C_{18}$ (Waters) 4.6 × 250 mm column and eluted with solvent A and B under a linear gradient mode to 80% acetonitrile over 25 min (flow rate = 1 ml/min). Hydroxyindole fraction was collected using TSK-GEL ODA-80TM (4.6 × 250 mm) under linear gradient mode to confirm the hydroxyl group position.

^{1}H-NMR Spectroscopy

NMR spectra were measured using Bruker DMX 500 MHz spectrometer. Samples were dissolved in CD_3OD from Nacalai tesque, Inc. (Kyoto, Japan).

Tandem Mass Spectrometry

All mass spectrometry experiments were carried out on a JEOL HX110A/HX110A four sector tandem mass spectrometer fitted with a FRIT/FAB probe, 6 kV Xe beam FAB gun and the MS-ADS11 variable mass dispersion array detector (JEOL, Akishima, Tokyo, Japan) [Ishihara et al., 1989]. Mass spectrometer was operated at a scan speed of 40–1,000 amu in 4 sec for LC/MS. FAB high resolution measurements were carried out using FAB double target MS-DTPM09 unit under the 5000 resolution. The CID spectra were recorded with 10 kV acceleration voltage (MS1), electrically 8 kV floated collision cell and (1:1.1) focal plane array detector conditions.

Reagents and Supplies

Glycerol for FAB matrix was obtained from Tokyo Kasei Co., Ltd. (Tokyo, Japan). High performance liquid chromatographic grade acetonitrile and distilled water (Nacalai tesque, Inc., Kyoto, Japan) were used for the preparation of the samples and mobile phases. All mobile phase solvents contained 0.1% TFA (Nacalai tesque, Inc.) and were degassed prior to use.

RESULTS AND DISCUSSION

Identifications and Characterizations of Acylpolyamines

In order to find out the applicability of FRIT FAB-LC/MS for the purpose of isolation and structure determination of acylpolyamines stored in the spider venom gland, the system sensitivity was checked, firstly by injecting 10 μl of 2 and 20 pmol/μl solution containing a mixture of model compounds 1, 2, 3, and 4 (JSTX-3, [DHPP-]JSTX-3, JSTX-1, and des-Asn-JSTX-3, respectively) (Fig. 2) into a μ-column LC/FAB/MS system (Fig. 1). FAB spectrum and mass chromatogram of 2 pmol/μl JSTX-3 are shown as an example in Figure 3a,b. All four model compounds showed intense protonated molecular ions and proved that the system can detect few pmol level of acylpolyamines. The LC/FAB/MS and MS/MS results of crude extracts from a *Nephilengys borbonica* venom gland showed 25 intense $(M+H)^+$ ions as major constituents (Fig. 4) or 40 compounds if it includes minor compounds for which their existence were recognized by mass chromatograms and HPLC retention times (Fig. 14a–d). The use of LC/MS system was essential in distinguishing minor components from broad overlapping major components. Among those protonated molecular ions, m/z 566, 573, 589, 816, and 958 corresponded with known compounds JSTX-3, nephilatoxin-8,-1,-5, and -6, respectively. However, the remaining protonated molecular ion mass numbers did not fit any previously known acylpolyamine molecular weights. NPTX followed by molecular weight was used for the names of the

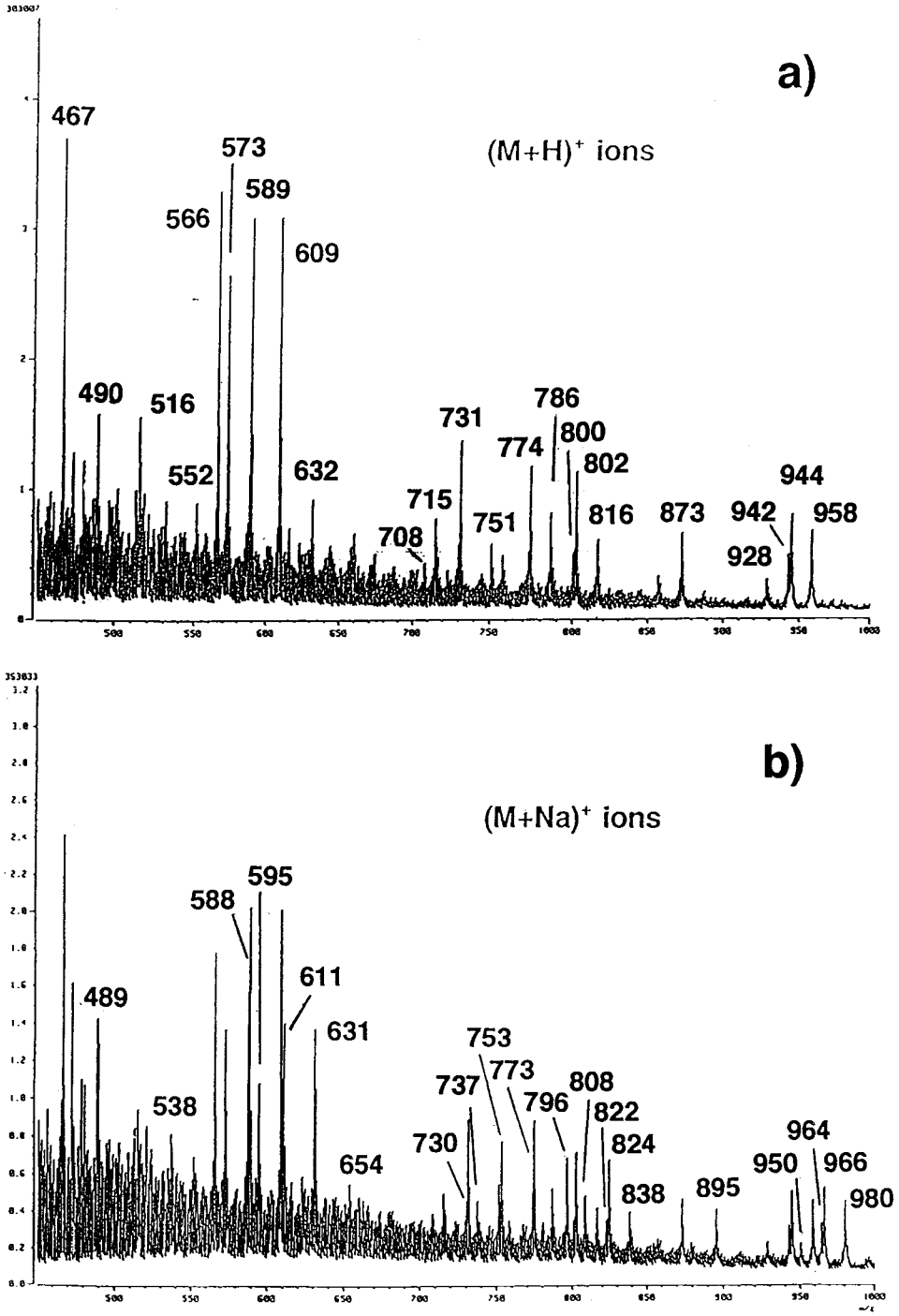

Fig. 5. a: FRIT FAB-MS spectrum of crude venom extracts. **b:** FRIT FAB-MS spectrum of sodium added crude venom extracts.

compounds unless structures were assigned as previously known compounds. FAB spectra of those compounds showed prominent protonated molecular ions but further structure interpretations were difficult because of weak fragment ion intensity and interfering FAB matrix signals. After detecting 25 major NPTX compounds by LC/MS, same crude extracts were submitted to flow injection mode FRIT-FAB/MS and FRIT-FAB/MS/MS measurements. The FAB spectrum of continuously flowing crude extracts showed that the ionization efficiency among those acylpolyamine type compounds were not suppressed significantly under the continuous flow FAB mode of operations (Fig. 5a). It suggested that the

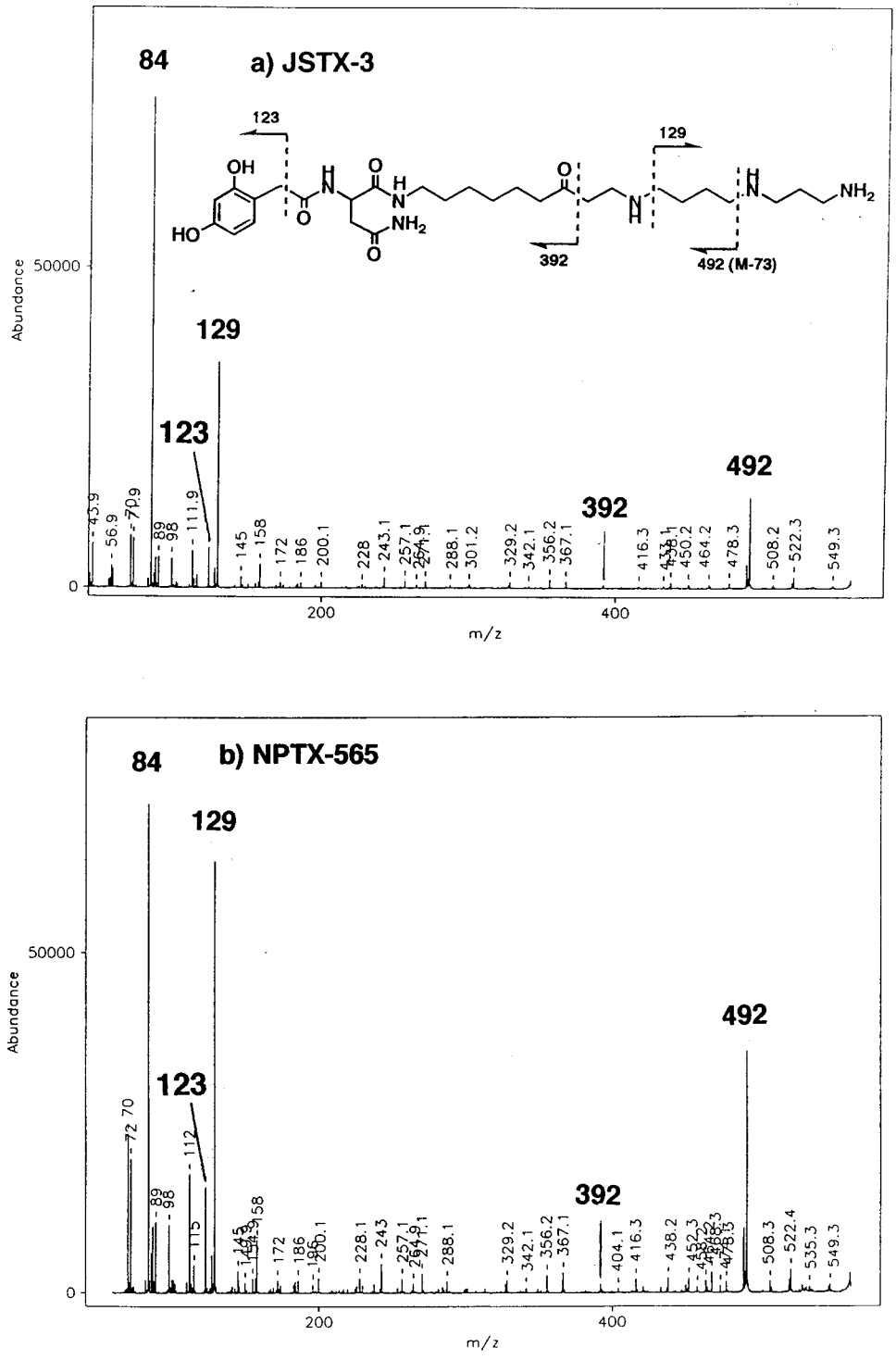

Fig. 6. CID spectra of protonated molecular ions of authentic JSTX-3 (**a**) and NPTX-565(JSTX-3) (**b**). **a:** Acyl moiety charge-retained product ions. **b:** Polyamine side chain charge-retained product ions from sodium attached NPTX-565(JSTX-3).

spider toxin acylpoyamines were subdivided into amino acid-containing and non-amino acid-containing groups [McCormick et al., 1993] but regardless of those classifications, all known spider acylpolyamines have one of the following aromatic groups as an acyl terminal, i.e., mono-, di-hydroxy phenyl-acetyl, indole-3-acetyl, or mono-hydroxyindole-3-acetyl. The CID spectra of NPTX-608, NPTX-714, and NPTX-588 showed m/z 123, m/z 130, and m/z 146 as

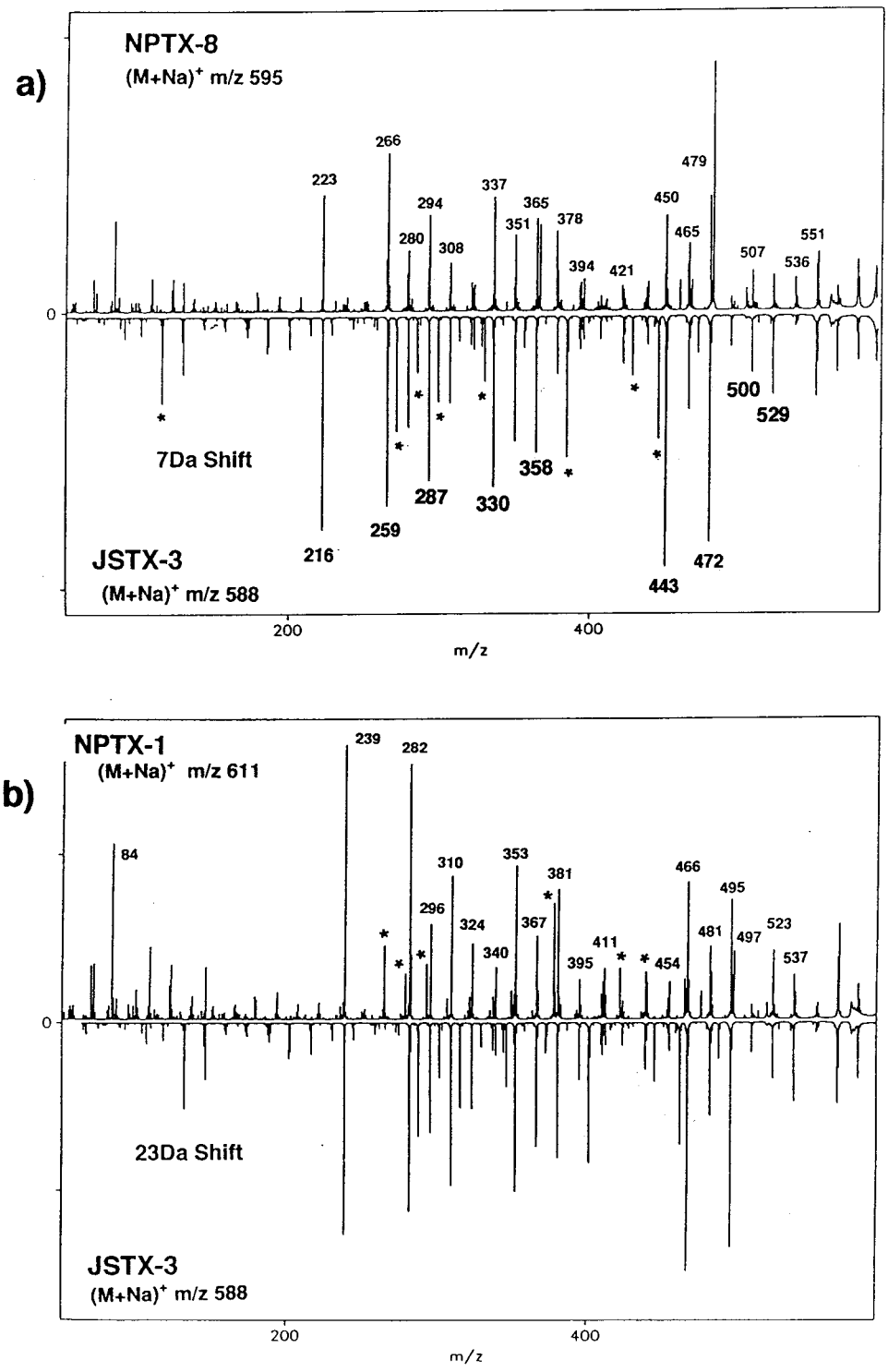

Fig. 7. Comparison of CID spectra from sodium-attached JSTX-3 m/z 588 and NPTX-8 (NPTX-572) (**a**), and NPTX-1 (NPTX-588) (**b**). The JSTX-3 spectrum has been plotted after +7 Da and +23 Da shift, respectively. * = Charge is retained on a polyamine chain side.

characteristic product ions, respectively, which could be correlated to dihydroxybenzene, indole and mono-hydroxy-indole and the ions corresponding with M-87 {-NH-$(CH_2)_4$-NH_2} and M-73 {-NH-$(CH_2)_3$-NH_2} were also found as the other side of terminal amine unit loss ions. Therefore, the existence of acylpolyamines in the crude extracts, the type of acyl group and the terminal unit of polyamine could easily be confirmed by the observation of the

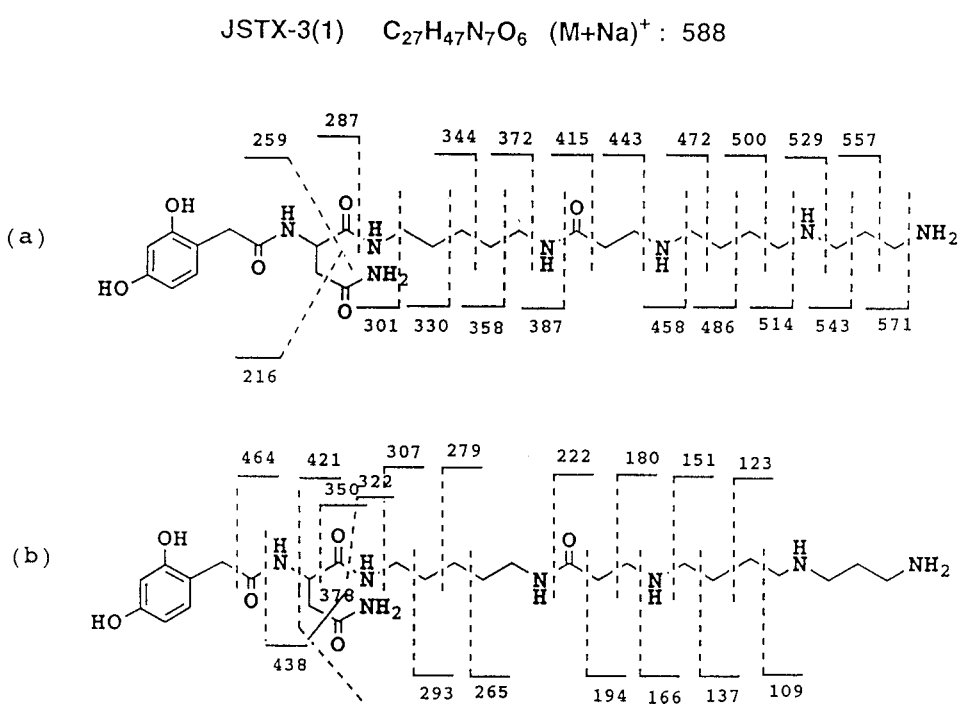

Fig. 8. a) Acyl moiety charge-retained product ions and **b)** polyamine side chain charge-retained product ions from sodium attached NPTX-565 (JSTX-3).

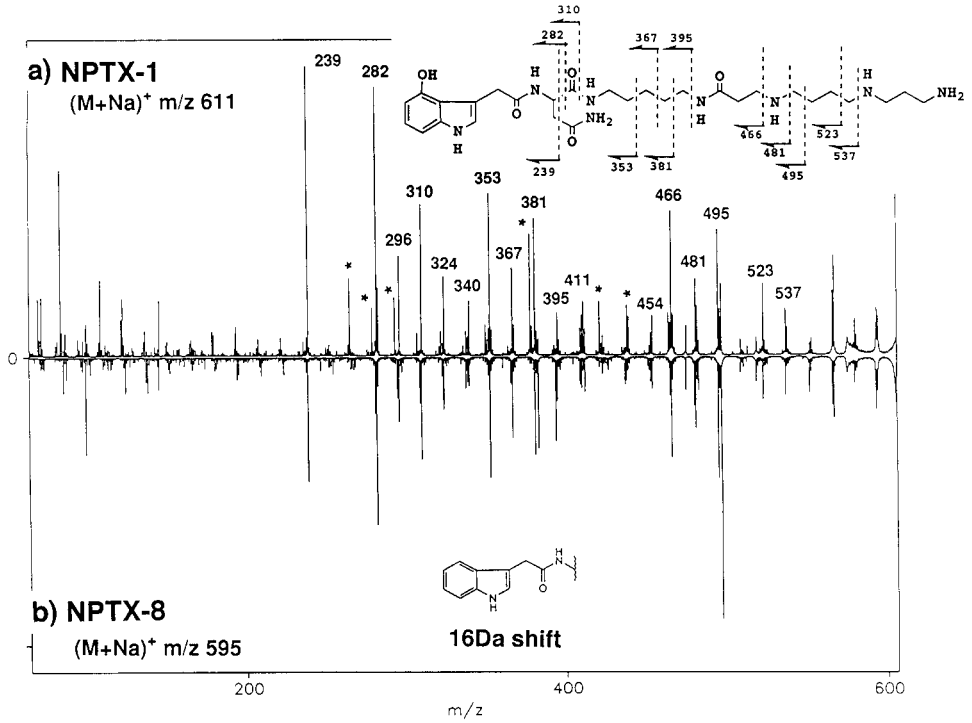

Fig. 9. Comparison of CID spectra from **a)** sodium-attached NPTX-1 m/z 611 (NPTX-588) and **b)** NPTX-8 m/z 595 (NPTX-572). The latter spectrum has been plotted after +16 Da shift.

CID spectra of $(M+H)^+$ ions. For further confirmations of the acyl moiety, UV absorption spectra of six chromatographically isolated acylpolyamines were examined and all observed UV spectra were divided into three absorption pattern groups which were characterized by Toki et al. [1992] as: a) di-hydroxy benzene λ_{max} at 210 and 280 nm; NPTX-466, NPTX-608 and NPTX-565, b) mono-hydroxyindole λ_{max} at 220, 265 280 nm; NPTX-588 and NPTX-773, and c) indole

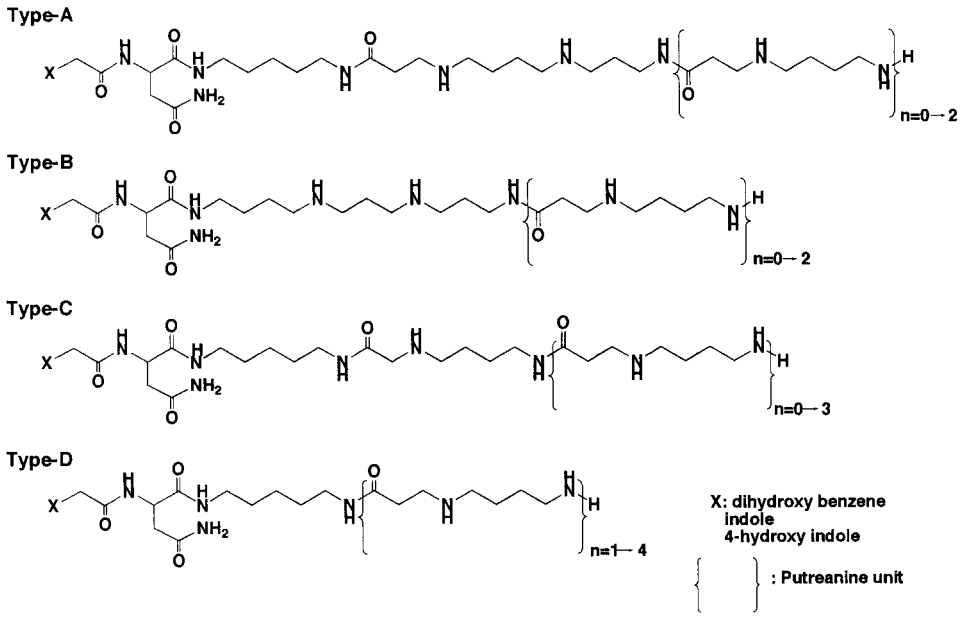

Fig. 10. Generalized structures type -A,-B,-C and -D of detected acylpolyamines from *Nephilengys boroonica* venom gland.

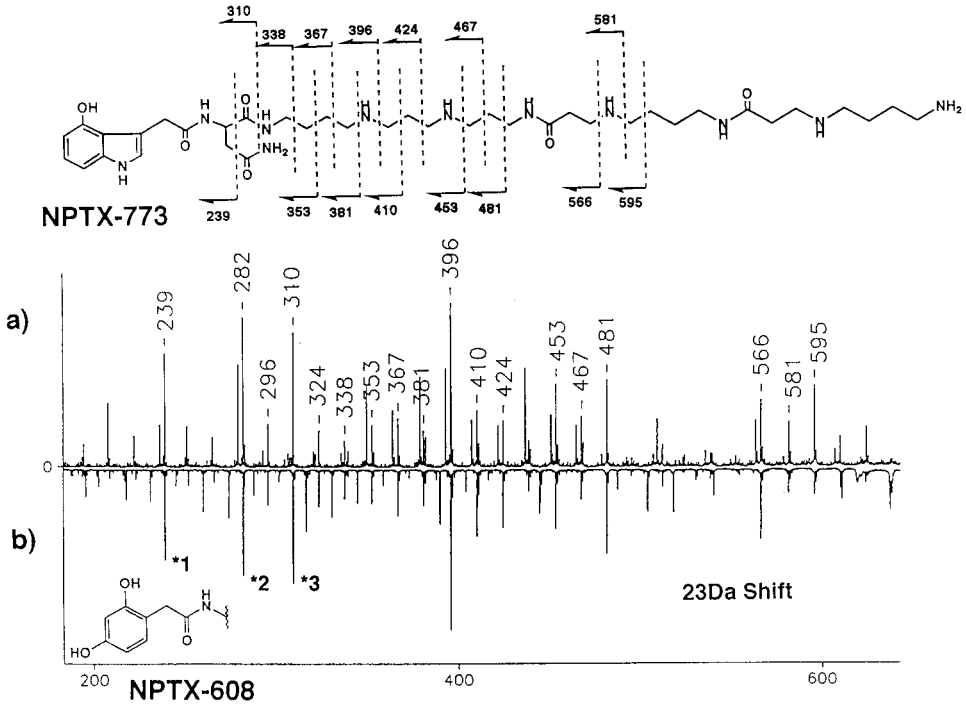

Fig. 11. Comparison of CID spectra from **a)** sodium-attached NPTX-773 and **b)** NPTX-608. The latter spectrum has been plotted after +23 Da shift. * 1, *2, and *3 are characteristic peaks of NPTX-608 lipophilic head moiety and original mass numbers are 216, 259, and 287, respectively.

λ_{max} at 220, 280, and 290 nm; NPTX-572. Previously, the hydroxyl group was placed at the 6-position of indole skeleton for nephilatoxins 1 to 6 on the basis of NMR observations [Toki et al., 1988].

But we have revised the hydroxyl group position from 6 to 4 based on the comparisons of NMR spectra of synthesized 4- and 6-hydroxyindole-3-acetic acid, HPLC separated hydroxyindole fractions and nephilatoxin-8 isolated from *Nephila clavata* venoms. The NMR spectrum of HPLC hydroxyindole fractions of *Nephilengys borbonica* venom extracts could also be assigned as 4-hydroxyindole (will be published elsewhere).

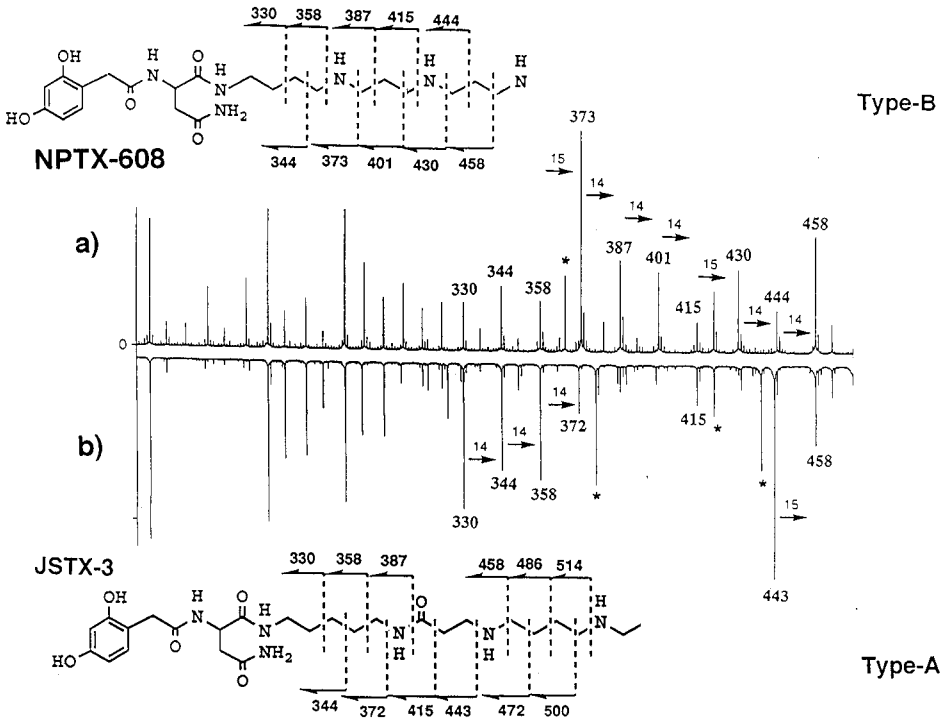

Fig. 12. Comparison of partial CID spectra from **a)** sodium attached NPTX-608 (type-B) and **b)** JSTX-3 (type-A).

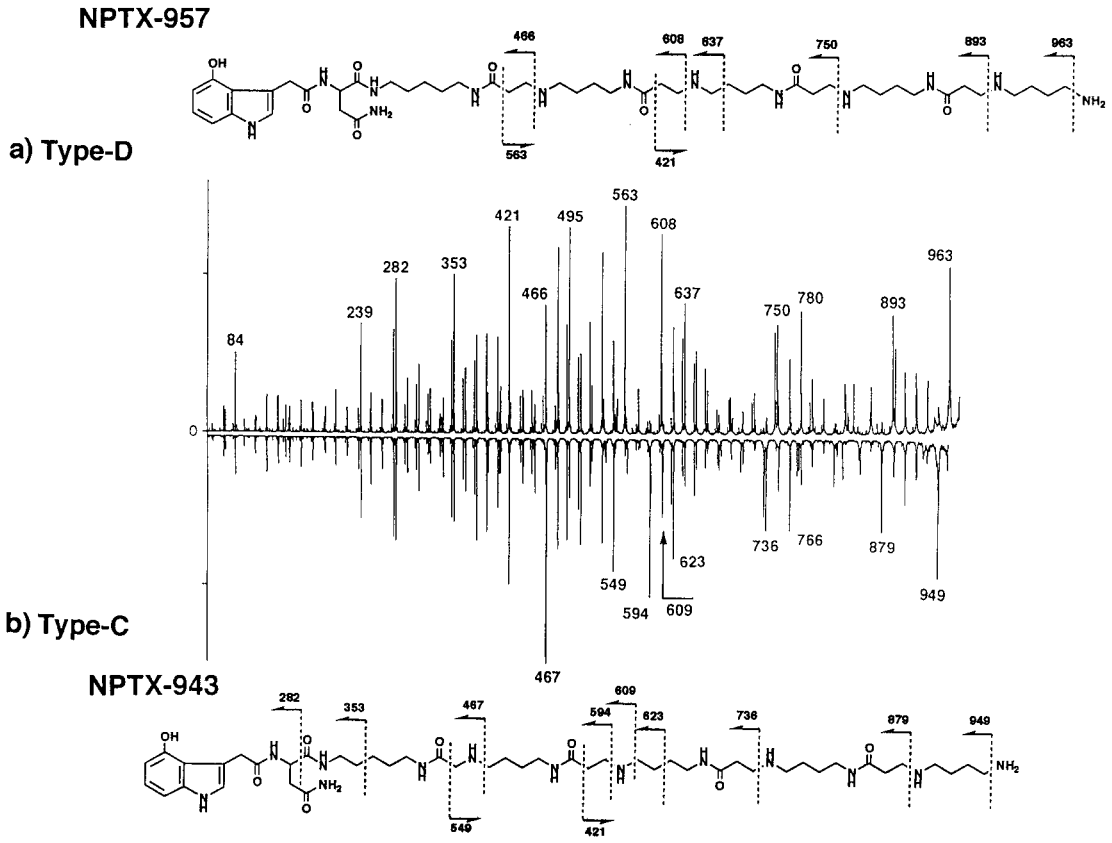

Fig. 13. Comparison of CID spectra from **a)** sodium-attached NPTX-957 (type-D) and **b)** NPTX-943(type-C).

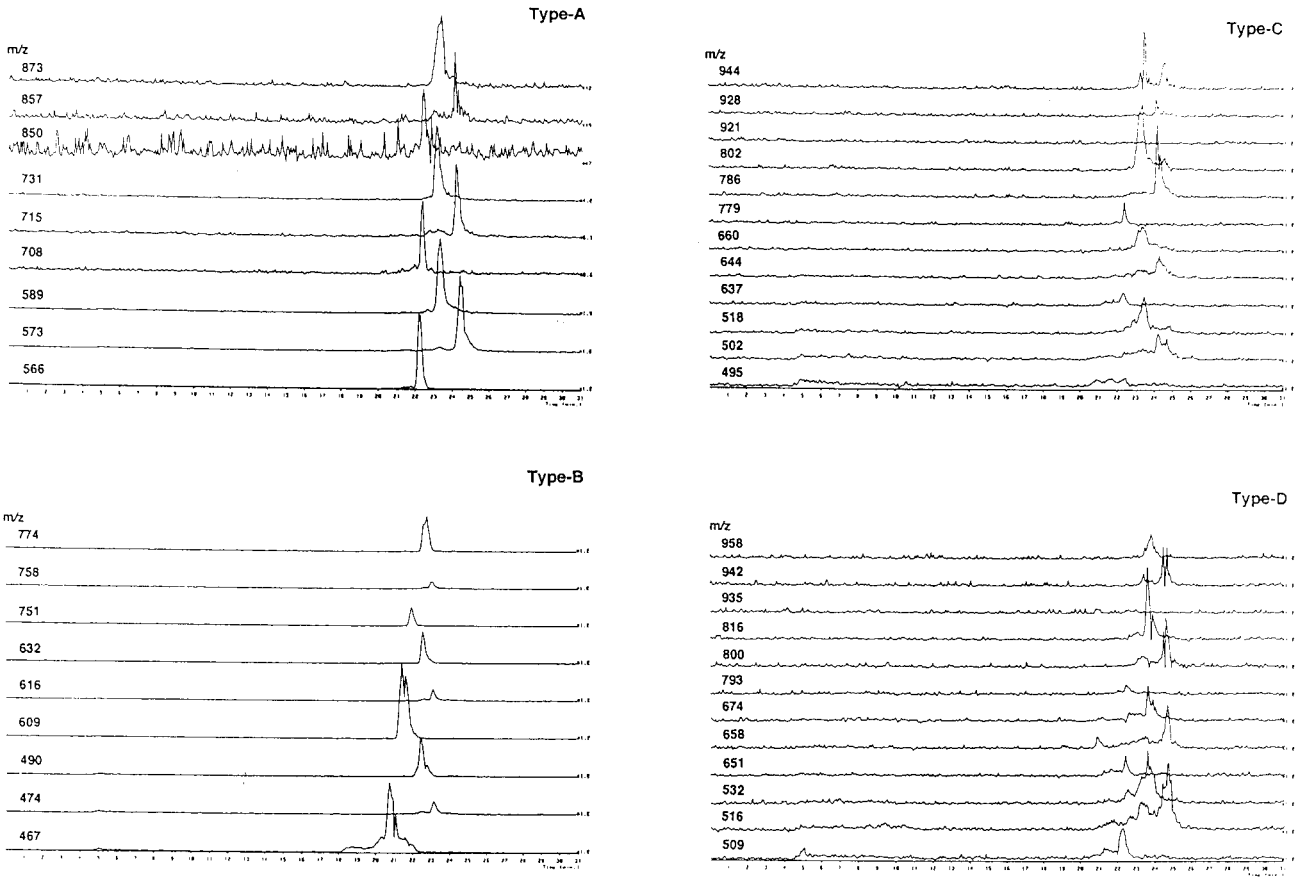

Fig. 14. Mass chromatograms of protonated molecular ions deduced from the combinations of acyl groups and numbers of putreanine: type-A , -B, -C, and -D.

Structure Determinations and Classifications of Acylpolyamines

The CID spectra of protonated molecular ions of authentic JSTX-3 and NPTX-565 showed good spectral agreement with each other including very weak signals (Fig 6a,b). Quistad et al. [1990] and McCormick et al. [1993] reported the CID analytical results of hydroxyl polyamine AG_{489} and a new calcium antagonist indole-3-acetoamide-3,4,3-polyamine. In those cases, the CID spectra clearly showed the locations of all nitrogen atoms in the polyamine chain. However, the CID spectra of asparagine containing JSTX-3 type acylpolyamines showed product ions originating predominantly from amino acid and terminal amine at low mass side. The product ions indicate that the nitrogen atom locations which Quistad and McCormick had observed, were weak and too obscure to assign. Therefore, the exact locations of amines and methylene chain length were not easily determined at low picomole level of samples. In a previous report, we have discussed charge remote fragmentation as a useful method for the characterizations of polyamine chains [Fujita et al., 1995]. The FRIT FAB spectrum of sodium added crude venom extracts showed clearly the sodium attached molecular ions $(M+Na)^+$ together with the $(M+H)^+$ ions (Fig. 5b). The CID spectra of $(M+Na)^+$ of JSTX-3(NPTX-565) is shown in Figure 7. Unlike the CID spectra of $(M+H)^+$, abundant sequence related product ions from polyamine chain were obtained by the effects of charge remote fragmentation. The product ions were compared with the previously reported fragmentations of authentic sample JSTX-3 (Fig. 8). All of the measured data of NPTX-565: the UV absorption spectrum, FAB high resolution exact mass number, and the CID spectra of $(M+H)^+$ and $(M+Na)^+$ allowed the structure of NPTX-565 to be assigned as a known compound JSTX-3.

From the observations of the CID spectra of all measured sodium attached acylpolyamines, at least one of the following intense characteristic product ion pairs formed by the cleavage of asparagine moiety were observed: 1) m/z 216, 259, 2) m/z 223, 266, and 3) m/z 239, 282 (Fig. 7a). This indicated that the detected acylpolyamines from *Nephilengys borbonica* venom gland have an acyl-asparagine connectivity which is a common structure of JSTX and Nephilatoxin series compounds. Mass number difference of compounds JSTX-3, NPTX-572[(JSTX-3) + 7 Da] and NPTX-588 [(JSTX-3) + 23 Da] suggested that those three compounds might have the same polyamine chain but different acyl groups; di-hydroxybenzene, indole, and monohydroxyindole, respectively. In the previous report, we

demonstrated that the difference of the acyl moieties shows no effects on the high-energy CID processes and that the product ions are produced with similar intensities. The CID spectrum of sodium attached JSTX-3 was shifted by 7 and 23 Da to higher mass side compared with the CID spectra of NPTX-8(NPTX-572) and NPTX-1(NPTX-588), respectively (Fig. 7a,b). In the same way, the actual profiling of the CID spectrum of m/z 611 and the 16 Da shifted CID spectrum of m/z 595 $(572+Na)^+$ are shown in Figure 9a,b. All the product ions retain the charge on the acyl group side were observed at the same mass number after the mass shift; this strongly suggests that while they have different acyl moieties, all three compounds have the same polyamine chain. Those three compounds were identified as JSTX-3 $(M+Na)^+$ m/z 588, nephilatoxin-8 $(M+Na)^+$ m/z 595, and nephilatoxin-1 $(M+Na)^+$ m/z 611 and were classified into general structure type-A (Fig. 10).

The CID spectrum of NPTX-608, characterized by a pair of ions m/z 216 and 259 ions, was shifted 23 Da to higher mass side compared with the NPTX-733 CID spectrum. The acyl group carrying product ions of NPTX-773 and NPTX-608 were observed at the same positions again with similar intensities (Fig. 11a,b) after a 23 Da shift (Type-B, Fig. 10). The 142 Da difference between NPTX-773 and NPTX-608 after the 23 Da shift corresponded to one putreanine unit. When the CID spectra of $(M+Na)^+$ ions of JSTX-3 (Type-A) and NPTX-608 (Type-B) were compared (Fig. 12a,b), both spectra gave exactly the same product ions up to m/z 358 including mass m/z 330 and 344, which indicates that those two compounds have the same di-hydroxybenzene and asparagine units at the acyl side. But the 15 Da difference between m/z 358 and 373 of NPTX-608 suggested the existence of -NH instead of a methylene unit. Observations of consecutive product ions m/z 387(-CH_2), 401(-CH_2), 415(-CH_2), 430(-NH), 444(-CH_2), and 458-(-CH_2) allowed elucidation of the partial structure of Type-B. On the other hand, JSTX-3 showed mass number m/z 372(-CH_2) instead of m/z 373(-NH) and did not give a series of CH_2 related product ions until m/z 458.

The compounds distinguished by a series of 14 Da molecular weight different compounds such as M.W. 494, 501, 517, 636, etc., and M.W. 508, 515, 531, 650, etc. were classified type-C and -D, respectively. As an example, the CID spectra of sodiated NPTX-943 and Nephilatoxin-6 (NPTX-957, M.W. 957) were examined to find out their difference (Fig. 13a,b). These two compounds showed similar CID spectra to each other except for intense 1 Da different product ions at m/z 467(NPTX-943) and m/z 466(NPTX-957) which could lead to the (−NH) and (−CH_2) difference between those two compounds keeping the same structures from acyl group to cadaverine moieties. Thus one could conclude that NPTX-943 has one methylene unit less structure than NPTX-6. For further evidence, the intense product ion at m/z 563(NPTX-957), which is characteristic of product ions carrying charge at acylpolyamine side with cleavage at the bond adjacent to carbonyl group, was shifted to 14 Da lower m/z 549(NPTX-943) (Scheme 1). All product ion mass numbers and intensity over m/z 608 (NPTX-957) matched with the product ions of NPTX-943 after a +14 Da (CH_2) shift, which again supports that type-C and -D have the same $\{putreanine\}_{1\text{-}4}$ connectivity. The glycine moiety which had not been reported previously for the acylpoly-

TABLE I. A List of Acylpolyamines Detected from a *Nephilengys borbonica* Venom Gland

Name	M.W	diOH-Bz	Indole	4-OH Indole	Type	(Ptn)*n*	Identified by
JSTX-3	565	○			A	0	CID
Nephilatoxin-8	572		○		A	0	CID
Nephilatoxin-1	588			○	A	0	CID
NPTX-707	707	○			A	1	MC
NPTX-714	714		○		A	1	CID
NPTX-730	730			○	A	1	CID
NPTX-849	849	○			A	2	MC
NPTX-856	856		○		A	2	MC
NPTX-872	872			○	A	2	CID
NPTX-466	466	○			B	0	CID
NPTX-473	473		○		B	0	MC
NPTX-489	489			○	B	0	MC
NPTX-608	608	○			B	1	CID
NPTX-615	615		○		B	1	MC
NPTX-631	631			○	B	1	MC
NPTX-750	750	○			B	2	MC
NPTX-757	757		○		B	2	MC
NPTX-773	773			○	B	2	CID
NPTX-494	494	○			C	0	MC
NPTX-501	501		○		C	0	MC
NPTX-517	517			○	C	0	MC
NPTX-636	636	○			C	1	MC
NPTX-643	643		○		C	1	MC
NPTX-659	659			○	C	1	MC
NPTX-778	778	○			C	2	MC
NPTX-785	785		○		C	2	CID
NPTX-801	801			○	C	2	CID
	920	○			C	3	
NPTX-927	927		○		C	3	CID
NPTX-943	943			○	C	3	CID
NPTX-508	508	○			D	1	MC
NPTX-515	515		○		D	1	MC
NPTX-531	531			○	D	1	MC
NPTX-650	650	○			D	2	MC
NPTX-657	657		○		D	2	MC
NPTX-673	673			○	D	2	MC
NPTX-792	792	○			D	3	MC
NPTX-799	799		○		D	3	MC
Nephilatoxin-5	815			○	D	3	CID
	934	○			D	4	
NPTX-941	941		○		D	4	MC
Nephilatoxin-6	957			○	D	4	CID

*diOH-BZ, 2,4-dihydroxy benzene; (Ptn)n, number of putreanine (0–4); CID, Collision Induced Dissociation Spectrometry; MC, Mass Chromatography.

amines found in the spider venoms was proposed for the first time for type-C compounds instead of **β**-alanine type structures, which is a common partial structure for type-D compounds (Fig. 10).

The CID spectra of synthesized glycine and **β**-alanine type compounds also showed characteristic product ions at m/z 451 and m/z 450, respectively (will be published).

All compounds were confirmed to have a common structure (asparagine-cadaverine) and were classified into the generalized structure type A, B, C, and D by the difference following the amine connectivity. From the generalized structures, it is possible to search analogous compounds which were not confirmed by MS/MS measurements. With the combination of three acyl groups and different numbers of putreanine, 42 protonated molecular ions were searched by mass chromatograms and retention times. Among the 42 possible combinations, 40 protonated molecular ion peaks were confirmed. All detected compounds are listed in Table I.

CONCLUSIONS

In the present study a sensitive isolation and structure determination method for spider toxin acylpolyamines using FRIT-FAB LC/MS and MS/MS was developed. The high-energy collision charge remote fragmentation method played an important role in the structure characterizations of those acylpolyamines. This method should be applicable to characterizing other compounds stored in various spider venom glands.

ACKNOWLEDGMENTS

We thank Professor Hiroshi Irie of Nagasaki University for the synthesis of glycine and **β**-alanine type acylpolyamines; Dr. Mario S. Palma, a visiting professor from Institute of Biosciences of Rio Claro-UNESP, SP, Brazil, for helpful discussions; and Mrs. Chitose Matsuda for NMR spectra measurements. This work was partly supported by grant-in-aid for scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

Adams J, Gross ML (1987): Tandem mass spectrometry for collisional activation of alkali-metal-cationized fatty acids: A method for determining double bond location. Anal Chem 59:1576–1582.

Adams J (1990): Charge-remote fragmentations: Analytical applications and fundamental studies. Mass Spectrom Review 9:141–186.

Ann Q, Adams J (1993): Structure-specific collision-induced fragmentations of ceramides cationized with alkali-metal ions. Anal Chem 65:7–13.

Aramaki Y, Yasuhara T, Higashijima T, Miwa A, Kawai N, Nakajima T (1987a): Chemical characterization of spider toxin, NSTX. Biomedical Res 8:167–173.

Aramaki Y, Yasuhara T, Shimazaki K, Kawai N, Nakajima T (1987b): Chemical structure of joro spider toxin (JSTX). Biomedical Res 8:241–245.

Caprioli RM, Fan T, Cottrell JS (1986): Continuous-flow sample probe for fast atom bombardment mass spectrometry. Anal Chem 58:2949–2954.

Cottrell JS, Evans S (1987): Characteristics of multichannel electrooptical Detection system and its application to the analysis of large molecules by fast atom bombardment mass spectrometry. Anal Chem 59:1990–1995.

Fujita T, Itagaki Y, Naoki H, Nakajima T, Hagiwara K (1995): Structure characterization of glutaminergic blocker spider toxins by high-energy collision charge-remote fragmentations. Rapid Commun Mass Spectrom 9:365–371.

Ishihara M, Kammei Y (1989): Ion optical considerations of a quadru-pole-octapole lens system varying mass range on a simultaneous detector. Rapid Commun Mass Spectrom 3:420–423

Ito Y, Takeuchi T, Ishi D, Goto M (1985): Direct coupling of micro high-performance liquid chromatography with fast atom bombardment mass spectrometry. J Chromatogr 346:161–166.

Jasys VJ, Kelbaugh PR, Nason DM, Phillips D, Rosnack KJ, Saccomano NA, Stroh JG, Volkmann RA (1990): Isolation, structure elucidation, and synthesis of novel hydroxylamine-containing polyamines from the venom of the *Agelenopsis aperta* spider. J Am Chem Soc 112:6696–6704.

Kassel DB, Williams KP, Musselman BD, Smith JA (1991a): Optimization of the fragmentation in a FRIT-fast atom bombardment ion source for the sequencing of peptides at the picomole level. Anal Chem 63:1978–1983.

Kassel DB, Musselman BD, Smith JA (1991b): Primary structure determination of peptides and enzymatically digested proteins using capillary liquid chromatography/mass spectrometry and rapid linked-scan techniques. Anal Chem 63:1091–1097.

Kim MS, McLafferty FW (1978): Efficiency of collisional activation of gaseous organic ions. J Am Chem Soc 100:3279–3282.

McCormick KD, Kobayashi K, Goldin SM, Reddy NL, Meinwald J (1993): Characterization and synthesis of a new calcium antagonist from the venom of a fishing spider. Tetrahedron 49:11155–11168.

McCormich KD, Meinwald J (1993): Nerotoxic acylpolyamines from spider venoms. J Chem Ecol 19:2411–2451.

Nagae N, Takeuchi T (1991): Rapid separation of proteins and peptides by reversed-phase microcolumn liquid chromatography. J Microcolumn Separations 3:5–9.

Pleasance S, Thibault P, Moseley MA, Deterding LJ, Tommer KB, Jorgenson JW (1990): Continuous flow fast atom bombardment with packed microcolumns: A comparison of precolumn versus coaxial matrix delivery. J Am Soc Mass Spectrom 1:312–319.

Quistad GB, Suwanrumpha S, Jarema MA, Shapiro MJ, Skinner WS, Jamieson GC, Lui A, Fu EW (1990): Structures of paralytic acylpolyamines from the spider *Agelenopsis aperta.* Biochem Biophys Res Commun 169:51–56.

Quistad GB, Reuter CC, Skinner WS, Dennis PA, Suwanrumpha S, Fu EW (1991): Paralytic and insecticidal toxins from the funnel web spider, *Hololena curta*. Toxicon 29:329–336.

Skinner WS, Dennis PA, Lui A, Carney RL, Quistad GB (1990): Chemical characterization of acylpolyamine toxins from venom of a trap-door spider and two tarantulas. Toxicon 28:541–546.

Toki T, Yasuhara T, Aramaki Y, Osawa K, Miwa A, Kawai N, Nakajima T (1988): Isolation and chemical characterization of a series of new spider toxin (nephilatoxins) in the venom of Joro spider, *nephila clavata.* Biomed Res 9:421–428.

Toki T, Yasuhara T, Aramaki Y, Hashimoto Y, Shudo K, Kawai N, Nakajima T (1990): Molecular structures of spider toxins (JSTX-1,2,3 and 4) in the venom of *Nephila clavata* L. Koch. Jpn J Saint Zool 41:9–14.

Toki T, Miwa A, Kawai N, Hagiwara K, Nakajima T (1992): Paralytic effect of spider toxin-related compounds on german cockroach. Biomed Res 13:53–58.